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14. ABSTRACT
Learning disabilities severely deteriorate the life of many NF1 patients. However, the pathogenic process for NF1-associated learning disabilities has not been fully understood and an effective therapy is not available. This study was proposed to identify genes that are dysregulated in the hippocampus of the Nf1+/- mouse model by DNA microarray analysis. Characterization of these NF1-affected genes will dramatically improve our understanding of the molecular pathogenesis underlying NF1-associated learning deficits. During the second year of the project, we have focused on comparing the genomic expression between wild-type and NF1 hippocampi to identified NF1-affected genes. We performed 5 independent DNA microarray experiments with wild-type and NF1 RNAs. Results from these microarray analyses indicated that many hippocampal genes are dysregulated in NF1 mice. We also performed bioinformatic analyses on the microarray data to investigate the particular neuronal processes are affected by NF1. We found that many of the affected genes are related to synaptic functions.

NF1, hippocampus, learning disabilities, LTP, microarray, gene

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Introduction

Learning disabilities severely deteriorate the life of many NF1 children by limiting their academic achievement, higher education and career choice (1). However, the pathogenic process for NF1-associated learning disabilities has not been fully understood and an effective therapy is not available. Drs Silva's and Zhong's laboratories have demonstrated that *Nf1* mutations lead to the development of learning deficits in mouse and *Drosophila*, respectively (2-4). Their work suggests that *Nf1* mutations cause learning deficits by disturbing the Ras/MAPK and/or cAMP signaling. Despite these significant progresses, NF1-affected downstream genes that directly contribute to deficits in synaptic plasticity and learning are largely unknown. We proposed to identify genes that are dysregulated in the hippocampus of the $Nf1^{+/-}$ mouse model. Characterization of these NF1-affected genes will dramatically improve our understanding of the molecular pathogenesis underlying NF1-associated learning deficits.

Body

During the second year of this project, we focused on following research activities outlined in the Statement of Work:

1. Completed microarray experiments on the NF1 hippocampus (Task 1). We purified RNA from the hippocampus of wild-type and NF1 mice, and performed 5 pairs of independent DNA microarray experiments, using Affymetrix GeneChips (MG-430). Genes that were significantly changed in NF1 hippocampi were identified by using the CyberT statistical analysis package for microarray data analysis (http://visitor.ics.uci.edu/genex/cybert/index.shtml). At the $p<0.01$ level, we identified 785 probe sets that are altered in the NF1 hippocampus. We performed real-time RT-PCR experiments to confirm our microarray results. Many of NF1-affected genes are upregulated, while others, including NF1, are downregulated (Fig. 1; Table 1). These genes are implicated in a variety of biological processes, including signal transduction, transcription and protein degradation.
2. Performed extensive bioinformatic analysis of the microarray data (Task 1). With the large number of NF1-affected genes identified by the microarray experiments, we next focused on data mining to determine the functions of individual genes. We used various bioinformatic techniques for this purpose, including gene ontology analysis, pathway analysis, and searching published databases. This data mining process took much effort and was very time-consuming. As a result of this analysis, we found many of the NF1-affected genes are implicated in synaptic function, indicating a disturbance of synapses in the NF1 hippocampus. For instance, a number of NF1-affected genes are involved in neurotransmitter release, including Rab3A, synaptotagmins and CASK (Fig. 2). Multiple NF1-affected genes are involved in postsynaptic functions; these genes include NMDAR1, AMPAR4 and mGluR5 (Fig. 3). In addition, several genes involved in regulation of synaptic structures such as Ncam1, integrins and neurexin1 are

dysregulated in the NF1 hippocampus (Fig. 4). Together, our microarray data suggest that synaptic structure and function are probably disturbed in the NF1 hippocampus. Such synaptic disturbances mediated by the dysregulation of NF1-affected genes likely contribute to the development of NF1 learning disabilities. These new findings lay down a foundation for further define the molecular etiology of learning deficits developed in NF1 patients.

3. Recommended changes or future work to better address the research topic: The Task 2 in the original proposal was to identify genes that are dysregulated during LTP expression in the NF1 hippocampus. After evaluating the progress we made in the past year and recent important advances in the field, the PI recommends to perform a new study that is considered to be with a higher priority than the original work described in Task 2. This recommended new study is to perform microarray analysis on NF1 mice treated with lovastatin. We propose this new study because a recent exciting study shows that lovastatin treatments of NF1 mice can reverse NF1-associated learning deficits (5). We reason that lovastatin would also reverse the expression of NF1-affected genes that underlie NF1-associated learning deficits. Therefore, by comparing the genomic expression patterns of the hippocampus of lovastatin-treated NF1 and sham-treated NF1 mice via microarray analysis, we will be able to identify the lovastatin-corrected genes. We will then compare the NF1-affected genes and lovastatin-corrected genes to identify the NF1-affected genes that can be corrected by lovastatin treatments. This research will dramatically help us narrow down the NF1-affceted genes that are critical for the development of NF1 learning disabilities. We believe that, compared to the work described in the Task 2, this is a better experiment for identification of genes and molecular pathways that are critical for the development of NF1 learning disabilities, which is the objective of this research. With the approval, we will mainly focus on this new research outlined above.

Key Research Accomplishments

1. Complete 5 independent microarray experiments to compare the genomic expression patterns of wild-type and NF mice.
2. Performed rigorous bioinformatic analyses of the NF1-affected genes to investigate the potential neuronal functions disturbed in the NF1 hippocampus.

Reportable Outcomes

On January 21, 2006, we presented our microarray studies at the NF1 symposium organized by the California NF, Inc. The PI will also participate in the Conference of NF1 Learning Disabilities organized by Children Tumor Foundation. We do not have manuscripts from this research yet, but expect to have when the project is completed. By performing the described research work, two trainees (one postdoctoral fellow and one young research technician) gained experiences in NF1 research.

Conclusions

In summary, we have successfully completed the DNA microarray analysis on the NF1 hippocampus. This analysis has led to the identification of a large number of NF1-affected genes in the hippocampus. Many of them are involved in the regulation of synaptic structures and functions. These progresses will facilitate the investigation of the molecular pathways that are disturbed in the NF1 hippocampus and underlie NF1-associated learning disabilities. To better address the problem of this research work, we also recommend performing microarray analysis on NF1 mice treated with lovastatin to identify lovastatin-corrected genes that are affected in the NF1 hippocampus. The NF1-affected genes that can be corrected by lovastatin are expected to be critical for the development of NF1 learning disabilities.

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Figure 1. Hierarchical clustering of NF1-affected genes identified by microarray analyses. Shown are a portion of NF1-affected genes ($p<0.01$). DNA microarray analyses were performed for five replicates for wild-type and NF1 mice, using RNA samples from different animals (Columns). The expression of NF1-affected genes is shown in individual rows. The left panel show the genes that are upregulated; the right panel are the genes that are downregulated (The NF1 gene is highlighted with green). A color scale is shown under each graph, with red to indicate high expression and green low expression.

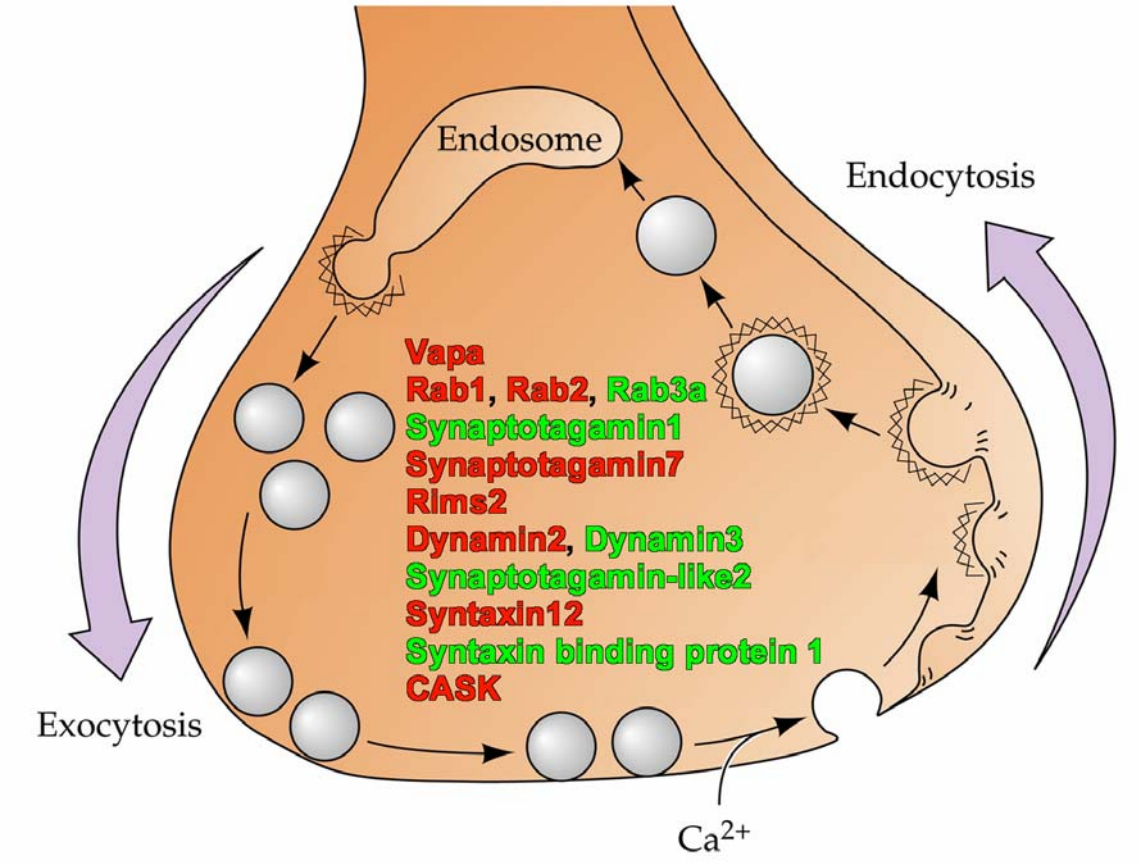

Figure 2. NF1-affected genes with pre-synaptic functions. NF1-upregulated genes are indicated by red names; NF1-downregulated genes are indicated by green names.

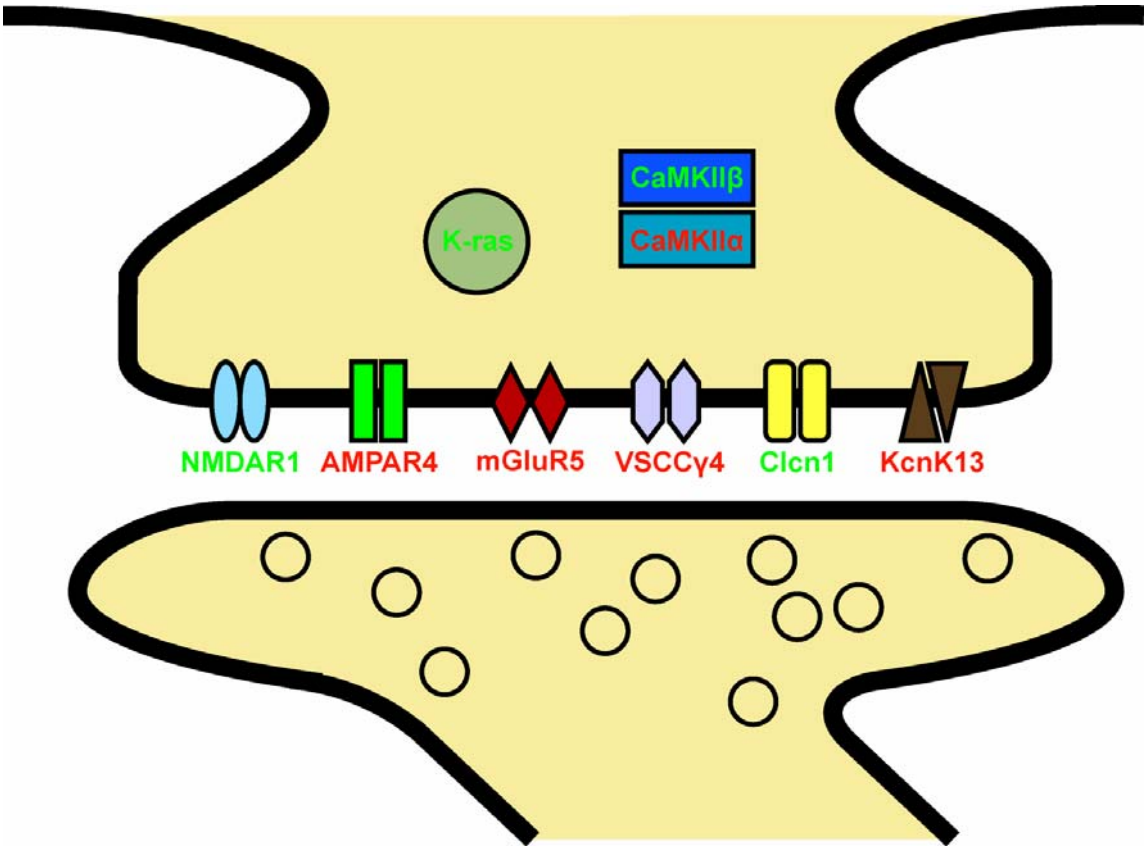

Figure 3. NF1-affected genes with post-synaptic functions. NF1-upregulated genes are indicated by red names; NF1-downregulated genes are indicated by green names.

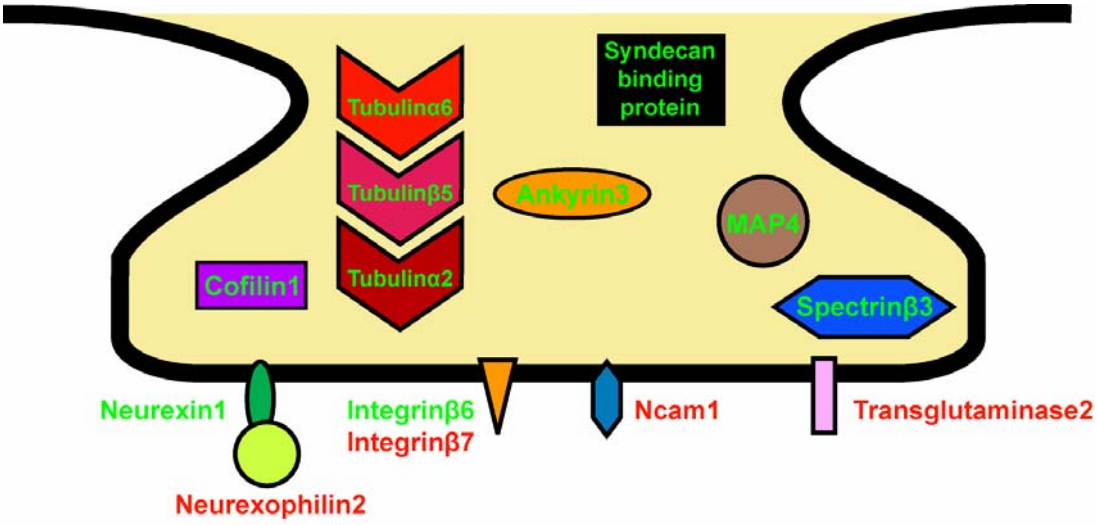

Figure 4. NF1-affected genes encoding structural proteins at synapses. NF1-upregulated genes are indicated by red names; NF1-downregulated genes are indicated by green names.

Gene Symbol	Gene Title	Up or Down Regulated
Adcy7	Adenylate cyclase 7 (Adcy7), mRNA	↑
Cacng4	calcium channel, voltage-dependent, gamma subunit 4	↑
Galnact2	Chondroitin sulfate GalNAcT-2 (Galnact2), mRNA	↑
Coasy	Coenzyme A synthase	↑
Ftl1	ferritin light chain 1	↑
Hmgb2	high mobility group box 2	↑
Irak3	interleukin-1 receptor-associated kinase 3	↑
Mettl5	methyltransferase like 5	↑
Map2k2	Mitogen activated protein kinase kinase 2 (Map2k2), mRNA	↑
Mapkapk3	mitogen-activated protein kinase-activated protein kinase 3	↑
Mtpn	myotrophin	↑
Kcnk13	potassium channel, subfamily K, member 13	↑
Rab28	RAB28, member RAS oncogene family	↑
Stk11ip	serine/threonine kinase 11 interacting protein	↑
Slc1a3	solute carrier family 1, member 3	↑
Stx12	syntaxin 12	↑
Tax1bp1	Tax1 (human T-cell leukemia virus type I) binding protein 1	↑
MGI:1929091	teratocarcinoma expressed, serine rich	↑
Tox	Thymocyte selection-associated HMG box gene (Tox), mRNA	↑
Tmie	transmembrane inner ear	↑
Ube3a	ubiquitin protein ligase E3A	↑
Zfp318	zinc finger protein 318	↑
Nr3c2	nuclear receptor subfamily 3, group C, member 2	↓
Capn6	calpain 6	↓
Card10	caspase recruitment domain family, member 10	↓
Cd8a	CD8 antigen, alpha chain	↓
Cd84	CD84 antigen	↓
Chchd8	coiled-coil-helix-coiled-coil-helix domain containing 8	↓
Cyp2j9	Cytochrome P450, family 2, subfamily j, polypeptide 9 (Cyp2j9)	↓
Xpo7	exportin 7	↓
Foxk2	forkhead box K2	↓
Ftcd	Formiminotransferase cyclodeaminase	↓
Igk-V8	Immunoglobulin lambda chain, mAb 667	↓
Ly6i	lymphocyte antigen 6 complex, locus I	↓
Nf1	neurofibromatosis 1	↓
Pip5k2b	phosphatidylinositol-4-phosphate 5-kinase, type II, beta	↓
Pbx3	Pre B-cell leukemia transcription factor 3 (Pbx3), mRNA	↓
Akt1	thymoma viral proto-oncogene 1	↓

Table 1. Representative NF1-affected genes ($p<0.01$).

Revised Statement of Work

A Functional Genomic Analysis of NF1-Associated Learning Disabilities
Shao-Jun Tang

Task 1. To identify genes that are abnormally expressed in the NF1 mouse hippocampus, Months 1-24:

a. Establish the breeding colony for NF1 mice.
b. Purify hippocampal RNA from wild-type control and NF1 ($Nf1^{+/-}$) mice.
c. Prepare cRNA targets.
d. Perform hybridization on oligonucleotide microarrays.
e. Perform statistical analysis to identify genes that are abnormally expressed in the NF1 hippocampus.
f. Perform RT-PCR to confirm the abnormal expression of selected genes in the NF1 hippocampus.
g. Perform bioinformatic analyses to annotate the functions of genes and to identify the biological pathways that are affected in the NF1 hippocampus.
h. Perform LTP experiments to determine the functional significance of selected genes in LTP expression.

Task 2. To identify NF1-affected genes that can be corrected by lovastatin treatments, Months 6-36:

a. Treat NF1 mice with lovastatin or sham solution.
b. Purify hippocampal RNA from NF1 mice that are treated with lovastatin and control NF1 mice that are treated with sham solution.
c. Prepare cRNA targets.
d. Perform microarray hybridization.
e. Perform statistical analysis to identify hippocampal genes that are significantly changed by lovastatin treatments.
f. Perform real time RT-PCR to confirm the expression of selected genes.
g. Identify NF1-affceted genes that are corrected by lovastatin treatments by comparing the NF1-affected genes identified from Task 1 and lovastatin-affected genes identified from a-e of this Task.
h. Perform clustering and bioinformatic analyses to annotate the functions of NF1 genes that are corrected by lovastatin treatments and to identify their biological pathways and neuronal processes.